Hepatic differentiation of human embryonic stem cells on microcarriers

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Abstract

Translation of stem cell research to industrial and clinical settings mostly requires large quantities of cells, especially those involving large organs such as the liver. A scalable reactor system is desirable to ensure a reliable supply of sufficient quantities of differentiated cells. To increase the culture efficiency in bioreactor system, high surface to volume ratio needs to be achieved. We employed a microcarrier culture system for the expansion of undifferentiated human embryonic stem cells (hESCs) as well as for directed differentiation of these cells to hepatocyte-like cells. Cells in single cell suspension were attached to the bead surface in even distribution and were expanded to 1 x 10^6 cells/ml within 2 days of hESC culture with maintenance of the level of pluripotency markers. Directed differentiation into hepatocyte-like cells on microcarriers, both in static culture and stirred bioreactors, induced similar levels of hepatocyte-like cell differentiation as observed with cells cultured in conventional tissue culture plates. The cells expressed both immature and mature hepatocyte-lineage genes and proteins such as asialoglycoprotein receptor-1 (ASGPR-1) and albumin. Differentiated cells exhibited functional characteristics such as secretion of albumin and urea, and CYP3A4 activity could be detected. Microcarriers thus offer the potential for large-scale expansion and differentiation of hESCs induced hepatocyte-like cells in a more controllable bioreactor environment.

1. Introduction

Among many organs and tissues for which cell therapies are being pursued, the liver is an organ that lacks effective therapies for liver failure. The liver is the largest organ in the adult body and has major functions in metabolism and detoxification, playing a role in protein synthesis, glucose metabolism and lipid metabolism. Existing treatments for liver failure are limited, and the only real cure is liver cell or organ transplantation. The shortage of donor cells and organs often makes it unreachable for a large number of patients. Hepatocytes isolated from liver and cultured *in vitro* have limited proliferation potential and often very quickly lose their functional attributes. Therefore, investigators are looking into the possibility to generate hepatocytes from progenitor cells or stem cells. However, any therapeutic application requires a large quantity of cells in the order of $>10^9$-10^{10} cells per treatment. In addition to the clinical need for hepatic cell transplantation, human hepatocytes attract attention from the pharmaceutical industry for drug toxicity and metabolism testing to better estimate acute and chronic toxicity, which should decrease the failure of drugs in clinical trials. The shortage of human livers also makes it difficult to use primary human hepatocytes for pharmaceutical purposes.

The cultivation of hepatic progenitor cells, immortalization of hepatocytes, and derivation of functional hepatocytes from stem cells are among the more prevalent approaches (Agarwal et al., 2008; Allain et al., 2002; Cai et al., 2000; Hay et al., 2008; Herrera et al., 2006; Wege et al., 2003). Especially, the progress in stem cell science has opened the possibility that many types of stem cells including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), pluripotent adult stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) can be used to produce hepatocyte-like cells (Zhang et al., 2012). Hepatic cells derived

from pluripotent stem cells (PSCs), which are capable of unlimited self-renewal have great potential to be such a steady cell source.

For stem cells to be widely applied to clinical and pharmaceutical fields, having a desired cell type as well as a sufficient supply of those cells should be fulfilled. The conventional method of expanding and differentiating PSCs in tissue culture plates is labor intensive and difficult to scale up. Employing a scalable reactor system can enable ample supply of differentiation competent or differentiated cells to meet such a clinical need. Various reactor systems have been investigated to support stem cell expansion and differentiation towards hepatocytes; rotary culture systems, perfusion culture systems, and stirred suspension systems have been employed for hepatic cell cultures (Matsumoto et al., 2008; Miki et al., 2011; Wang et al., 2012; Yin et al., 2007). Among those systems, stirred suspension culture has the advantage of having been widely used in growing mammalian cells for decades. It also allows high-density culture in a more homogeneous environment, and should allow one to monitor and control culture parameters. Cells are suspended in culture either as spheroids or with the support of scaffold. Stirred reactor cultures have been used to expand or differentiate hematopoietic stem and progenitor cells (HSPCs), neural stem cells (NSCs), and human and mouse embryonic stem cell (ESCs) grown as embryoid bodies (EBs) (Cameron et al., 2006; Fok and Zanstra, 2005; Gilbertson et al., 2006; Li et al., 2006).

Microcarriers have also been used as scaffold to support differentiation in stirred suspension culture. Microcarriers are particles, typically made of cellulose, glass, plastic, or polyester, and usually with diameters of 100-250 μm. Microcarriers have the advantage of providing a large surface area for a given reactor volume. By providing surface for attachment, they allow anchorage dependent cells to be cultivated in stirred bioreactors for a large-scale.

Undifferentiated PSCs were seeded onto the beads, expanded further, and differentiated into different cell types, including neural progenitor cells and cardiomyocytes (Bardy et al., 2013; Lecina et al., 2010; Rodrigues et al., 2011). Few studies have assessed hepatic differentiation on microcarriers. Rat multipotent adult progenitor cells (rMAPCs) have been expanded and differentiated on dextran beads in a spinner system to yield hepatocyte-like cells expressing early and mature hepatic genes (Park et al., 2010). hESCs have also been expanded on different types of beads and differentiated into endodermal cells (Lock and Tzanakakis, 2009), showing the potential of scaled-up culture of ESC hepatic differentiation using microcarrier-based suspension culture. Although most micro-porous microcarrier cultures are still 2D, the stirred bioreactor allows more dynamic culture environment than in conventional 2D culture in tissue culture plates. In this study, using an established hepatic differentiation protocol (Roelandt et al., 2012; Roelandt et al., 2010), we show that hESCs can be differentiated into hepatocyte-like cells on dextran microcarriers in suspension culture system. Microcarrier culture has the potential of being an efficient scalable means of undifferentiated hESC expansion for a short period and directed differentiation towards hepatocytes.

2. Materials and methods

2.1. hESC culture

The human H9 cell line was used in this study, with approval of the ethics committee at KU Leuven. H9 cells were cultured in a feeder free condition on hESC qualified Matrigel™ (BD) coated surface and with mTeSR1™ (Stem cell technologies) medium.

2.2. hESC cell seeding onto microcarriers for suspension culture

Cytodex, a crosslinked dextran microbead, is microporous, with a void fraction greatly exceeding 90%, and supports cell growth on the external surface of the bead. Cytodex 1 and Cytodex 3 microcarriers were purchased from GE Healthcare as dry powder. Collagen based microcarriers, Cultispher and Spherecol, were tested for cell seeding and differentiation. Cultispher S (Sigma) are macroporous gelatin microcarriers. Spherecol® (Advanced BioMatrix) are purified collagen beads. The microcarrier stock was prepared by washing 1 g of microcarrier powder with PBS for 3-5 times. Swollen beads were sedimented in 100 ml fresh PBS and sterilized by autoclaving.

Prior to seeding the hESC, microcarriers were washed with DMEM/F12 (Life technologies) twice. Matrigel with reduced growth factors (BD) was diluted in DMEM/F12 in 1: 5 and added to the washed beads to make a final dilution 1:10. Beads were stirred in the Matrigel solution for 2 hours at 37°C. hESC cultures in 6 well plates (Corning) were washed with PBS. 1 ml Accutase (Sigma) was added to each well, and the plates were incubated at 37°C for 5 min. The plates were lightly tapped several times and gently pipetted to make single cell suspension. 2 ml of DMEM/F12 was added to each well to neutralize accutase. The cells were collected, centrifuged, and resuspended in mTeSR1 medium at a concentration of $5x10^6$ cells/ml. Cells were added to

25 mg/ml Cytodex microcarriers at the concentration of 5 x 10^6 cells/ml in 5 ml culture medium. The mixture was incubated for 2 hrs at 37°C to allow initial cell attachment to the beads with occasional shaking of the tube. Then the mixture was transferred into either a low attachment plates (Corning) or a spinner flask (Bellco). The culture was stirred at 20-25 rpm on a magnetic stir plate (2mag) and maintained at 37°C, 21% O_2 tension and 5% CO_2 for 2-3 days of expansion with medium change every day. 90% of medium was removed carefully when cell-laden beads were sedimented, and then 100% of fresh medium was added to the flask.

2.3. In vitro Hepatic differentiation

hESC were differentiated into hepatocyte-like cells using a four step hepatic differentiation protocol developed in Verfaillie lab (Roelandt et al., 2012). For static plate differentiation, cell laden beads were inoculated in an ultra-low attachment 6-well plate (Corning) at a starting cell concentration of 1.0 x 10^6 cells/ml. After 2 days of expansion culture, the cells reached confluency on the microcarrier surface, they were washed once with PBS and resuspended in hepatic differentiation medium (2 ml of differentiation medium per well for static differentiation and 50 ml in a 250ml spinner flask for stirred suspension differentiation). The composition of serum free differentiation basal medium is a 60/40 (v/v) mixture of low glucose Dulbecco's Modified Eagle media (DMEM) (Life technologies) and MCDB-201 (Sigma) supplemented with 0.026 µg/ml ascorbic acid 3-phosphate (Sigma), linoleic acid bovine serum albumin (LA-BSA, Sigma) (final concentrations of 0.25x10^3 µg/ml BSA and 2.03 µg/ml linoleic acid), insulin-transferrin-selenium (ITS, Sigma) (final concentration 2.5 µg/ml insulin, 1.38 µg/ml transferring, 0.0012 µg/ml sodium selenite), 0.4 µg/ml dexamethasone (Sigma), 4.3 µg/ml β-mercaptoethanol (Sigma). The growth factor supplements were added with the 90% medium change as follows: (i) day 0: Activin A (100 ng/ml) and Wnt3a (50 ng/ml); (ii) day 2: Activin A (100 ng/ml); (iii)

day 4: BMP4 (50 ng/ml); (iv) day 8: aFGF (50 ng/ml); (v) day 12: HGF (20 ng/ml). Differentiations were carried out at 21% O_2 and 5% CO_2, 37°C for 18 days with 60% media change every 2 days corresponding to the differentiation stage.

2.4. RNA isolation and quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell lysates using the RNAeasy minikit (Sigma) according to instructions provided in the kit. cDNA was synthesized from the extracted RNA using the Super-script III reverse transcriptase (Life technologies) method. The PCR mix consisted of cDNA samples, SYBR Green Mix PCR reaction buffer (Life technologies) and primers (5 µM stocks, sequences in Table 1). The RT-qPCR reaction was run on a Realplex mastercycler (Eppendorf) using the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min followed by a dissociation protocol to obtain a melting curve. Transcription abundance relative to *GAPDH* was calculated as Δ Ct which is Ct (transcript of interest) – Ct (GAPDH) and transcript abundance in sample relative to control differentiation was calculated as Δ Ct (control) - Δ Ct (day of sample).

2.5. Cell viability staining

The live/dead viability/cytotoxicity kit (Life Technologies) was used to stain cells on microcarriers with calcein and ethidium. Component B was added first to DPBS (1:1000) and then component A was added to the solution (1:1000). Cells were incubated with the staining solution for 15-30 min at 37°C. Cells were washed once with PBS and observed under inverted fluorescent microscope (Axiovert, Zeiss). Live and dead cells appear as green and red respectively.

2.6. Albumin and ASGPR-1immunostaining

Cell-laden microcarriers were fixed with 10% NBF for 15 min and washed with PBS 5 min each 3 times. Fixed samples were treated with PBS with 0.2% Triton X-100 (PBST) for 10 min and blocked in 5% Goat serum in PBST for 1 h. Cell-laden-beads were stained with either an anti-asialoglycoprotein receptor-1 (ASGPR-1) antibody (Thermoscientific, 1:10) or anti albumin antibody (Dako, 1:4000) in DAKO antibody diluent at 4°C for overnight and stained with Alexa488 anti-rabbit IgG or anti-mouse IgG (Life Technologies, 1:500) for 30 min. Cells were counter stained for nuclei with Hoechst (1:2000). Then cells-microcarriers were washed, resuspended in PBS, and observed under inverted fluorescent microscope (Axiovert, Zeiss).

For flow cytometric analysis, cells were harvested from the microcarriers by trypsinization. After fixing with 4% PFA for 20 min and blocking for 1h in PBS with 3% (v/v) FBS, cells were incubated with anti ASGPR-1 antibody (1:10) for 30 min, followed by Alexa 488-conjugated goat anti-mouse IgG secondary antibody (1:500) for 30 min. Then cells were washed and resuspended in 500µl PBS for flow cytometry analysis using a FACS Canto (Becton Dickinson).

2.7. Albumin and urea secretion assay

The albumin secretion rate was quantified using the human albumin ELISA quantitation kit (Bethyl) according to instructions provided in the kit. Urea production rate was measured by using QuantiChrom urea assay kit (BioAssay Systems) following the instructions provided in the kit for low urea samples.

2.8. CYP3A4 activity assay

CYP3A4 activity was quantified using either P450-Glo™ CYP3A4 assay with Luciferin-IPA kit (more selective substrate for CYP3A4) or Luciferin-PFBE (commonly used in the published

literature, but which measures general CYP3A activity aside from CYP3A4) (Promega) according to instructions provided in the kit.

2.9. Cell enumeration by nuclei counting

Microcarriers from 1 ml culture were centrifuged at 1000 rpm for 30s to remove supernatant. Cells-microcarriers were washed once with PBS and treated with Liberase™ for 30-60 min. Cells were detached from the microcarriers by gentle pipetting to make single cell suspension. Cells were separated from the beads using a 100 µm filter and diluted for cell counting using Nucleocounter (Chemometec).

For fixative staining, cells on microcarriers in 1 ml were stained by adding a drop or two fixative staining solution (0.5% (w/v) crystal violet in 40% ethanol and 60% PBS) for 1-2 min. Stained cell-microcarriers were washed with PBS twice to remove the staining solution from the supernatant.

2.10. Statistical analysis

Experiments were repeated at least 3 times for each condition. Data from representative experiments are presented; whereas similar trends were seen in other multiple trials. All error bars represent standard deviation.

3. Results

3.1. hESC expansion and maintenance of pluripotency on microcarriers

hESCs are often cultured on mouse embryonic feeders, and passaged as small colonies for expansion. This makes it difficult to control the exact number of cells and the distribution of cells in tissue culture plates, and even more so for microcarrier cultures. To solve this problem, we isolated hESCs, cultured in mTeSR1 on hESC expansion Matrigel, as single cell suspension prior to seeding in plates or on microbeads for differentiation. hESCs, seeded as single cells and with a defined concentration (1.3×10^5 cell/cm^2), were allowed to attach to growth factor reduced differentiation Matrigel coated microcarriers or culture plates in mTeSR1 medium for 2 days before we started differentiation. In conventional differentiation using tissue culture plates, following standard differentiation, gene expression profile of hepatic markers and functional assay, in particular CYP3A4 activity was comparable between differentiations starting with single cell suspensions or with colonies (Fig. 1).

Different types of microcarriers with various materials including dextran beads with either positive charge or collagen layer (Cytodex 1 and Cytodex 3), collagen beads (Spherecol), and gelatin beads (Cultispher) were screened to select microcarriers for hESC hepatic differentiation. The expression levels of hepatic markers were higher in cells differentiated on dextran beads. Attached cells were clearly visible on the transparent dextran beads. Based on the hepatic gene expression level and the ease of cell-bead handling, Cytodex 3 was chosen for further analysis for hESC differentiation to hepatic cells (Fig. 2).

hESCs, isolated as a single cell suspension, were inoculated onto microcarriers at a seeding densities of 1.25×10^5, 2.5×10^5, 4.0×10^5, and 8.0×10^5 cells/mg bead and cultured for 3 days

to determine the optimal seeding density to reach confluent cell density after 2 days of expansion culture. With a seeding density of 8.0 x 10^5 cells/mg bead, cell density reached confluence after 2 days of expansion while lower seeding densities did not reach confluence after 3 days (Supplementary Table 1 and Fig. 3).

The total number of cells was inoculated onto beads at high concentration in 2-3 ml culture medium and incubated at 37°C for 2 hours with intermittent shaking every 5 minutes in order to increase the frequency of direct contact between microcarriers and cells. At this initial seeding density, many cells attached to beads in even distribution within 2 hours. The attachment efficiency was about 25%. The cell-laden microcarriers were then transferred into either low attachment 6-well plates or a 100 ml spinner flask with a 50 ml working volume of mTeSR1 medium at a final density of 8 x 10^5 cells/mg bead. After the initial 2 days in culture, viable hESCs covered the majority of the bead surface, and no empty beads could be detected (Fig. 3A and 3B).

When the cell concentration increased to 1.0 x 10^6 cells/ml, a plateau was reached (Fig. 3C) and the cell concentration or cell density on the beads did not increase any further, due to surface area restriction. By adjusting the initial seeding density to 8 x 10^5 cells/mg bead, we could control the time required to ensure that cells on the microcarrier surface became confluent, i.e. after two days following the seeding. The population doubling time on the microbeads was about 31h between day 0 and day 2, similar to that of H9 cell doubling in culture plates. As the cells became confluent on the beads, cell-laden beads started to agglomerate, but cells did not form multi-layers on the surface of the beads.

The expression levels of key hESC transcripts, octamer-binding transcription factor (*OCT4*), SRY (sex determining region Y)-box 2 (*SOX2*), and *NANOG* were assessed by RT-qPCR at day 0 (which also can represent the gene expression level of cells cultured in tissue culture plates) and day 2. Pluripotency gene expression was sustained at high levels during hESC proliferation (Fig. 3D). This demonstrates that the seeding method used prior to the expansion of hESC on microcarriers allows hESCs to become evenly distributed and achieve cell-confluency on microcarriers with maintained pluripotency during 2 day expansion culture.

3.2. Hepatocyte differentiation of hESC on microcarriers

3.2.1. Differentiation in static culture

The feasibility of employing microcarriers for hESC differentiation to hepatocyte-like cells was first investigated in static culture. Cells were expanded on microcarriers for 2 days to generate a confluent monolayer of cells. mTeSR1 medium was then replaced with differentiation medium containing growth factors as described in the materials and method section. Cell density decreased during step 1, slightly increased until day 12 (by morphological observation, data not shown), and then an overall decrease was seen to a final concentration of 3.8×10^5 cells/ml on day 18, which is similar to the cell number recovered from cells allowed to differentiate in tissue culture plates (Fig. 4A). As the differentiation proceeded, cell morphology changes were similar to those seen in hepatic differentiation in 2D on tissue culture plates, and the cells remained highly viable (Fig. 4B). As cells proliferated on microcarriers, cell-microcarriers form bigger bead-bead agglomerates allowing cells to grow in between the beads.

The level of hepatocyte-specific transcripts was evaluated at the end of the differentiation in microcarrier culture, and compared with cells cultured in culture dishes. Hepatocyte related

transcripts of alpha-feto-protein (*AFP*), albumin (*ALB*), glucose-6-phosphatase (*G6P*), microsomal glutathione S-transferase 1 (*MGST1*), *FACTOR V*, hepatocyte nuclear factor (*HNF4α*), cytochrome P450 family 3 subfamily A polypeptide 4 (*CYP3A4*), sodium/bile acid cotranspoter Na/Taurocholate cotransporting polypeptide (*NTCP*), orosomucoid 1 (*ORM1*), multidrug resistance-associated protein 2 (*MRP2*), glutathione S-transferase alpha (*GSTα*), phosphoenolpyruvate carboxykinase 1 (*PEPCK1*) were expressed at the end of differentiation (day 18) (Fig. 4C and 4D), with the averaged expression level of the hepatocyte marker transcripts from three replicate experiments comparable to that seen in differentiations carried on culture plates. Immunostaining for ALBUMIN, asialoglycoprotein receptor-1 (ASGPR-1), CYP3A4/5/7, and PEPCK identified regions of positive cells on the microcarriers (Fig. 5A). By flow cytometry, about 20% of the cells expressed ASGPR-1 (Fig. 5B), which is a type II transmembrane glycoprotein expressed on mature hepatocytes. As expected from the hepatic differentiation in tissue culture plates in previous studies, about 20% of cells were directed to hepatocytes with mature markers. The rest of the differentiated cell population consists of mostly immature hepatocytes and immature cells of other lineages (ex. Mesodermal cells expressing *FLK1*). We addressed how many cells also had endothelial and hepatic stellate cell features in the final differentiated population, but there was negligible expression of *CD32b*, *CD31* and *GFAP*.

Microcarriers have a high surface to volume ratio allowing cell culture with high cell concentration. We therefore tested if hepatic differentiation on microcarriers is feasible at higher cell concentration than conventional differentiation in tissue culture plates, which will be required in up-scaled culture system. Cell concentration at the beginning of differentiation was increased 2 times and 4 times respectively. Compared to differentiation in conventional tissue

culture plates, the cell yield from microcarrier cultures reached a 3 and 6 times higher final cell concentration (1.3×10^6 and 2.5×10^6 cells/ml), respectively, compared with tissue culture plate cultures at the end of differentiation (Supplementary Fig. 1A). Cells differentiated at higher concentration showed comparable levels of hepatic differentiation by gene expression and albumin secretion rate (Supplementary Fig. 1B and 1C).

3.2.2. Differentiation in stirred suspension culture

Next, hESC were expanded on microcarriers in a spinner flask and subsequently differentiated to the hepatocyte lineage in a spinner flask to confirm that the static microcarrier culture can be translated to a larger-scale bioreactor system. hESC were seeded on microbeads as described before. Upon reaching confluence, the hESC-laden microcarriers were washed with PBS once and resuspended in differentiation medium as per the differentiation protocol. With a stirring rate above 30 rpm, most cells detached from the microcarriers, and there was increased cell loss in general during the early phase of differentiation, which led to many void beads making it impossible to continue the culture (Supplementary Fig. 2). Using a stirring rate of 20-25 rpm, beads still agglomerated together, but at a lesser degree than in the static culture. The final concentration of cells at the end of differentiation was 5.9×10^5 cells/ml (Fig. 6A). The morphological changes of the cells on the beads cultured in the stirred bioreactor were similar to those seen in static culture. Throughout the differentiation on microcarriers in the stirred bioreactor, cells retained high viability (Fig. 6B). Transcripts for immature hepatocyte genes *AFP* and *HNF4α* were expressed at a high level. Transcripts for the more mature hepatic genes, *ALB, NTCP, ORM1, G6P, MGST1, FACTOR V, MRP2, PEPCK1, GSTα, CYP3A4* increased significantly by day18 of the hepatocyte differentiation (Fig. 6C). The expression level of mature transcripts was comparable to that in static microcarrier differentiation or differentiation on

tissue culture plates. Regions of positive cells were stained for ALBUMIN and ASGPR-1 (Fig. 6D).

3.2.3. Functional characteristics of the differentiated cells

In static microcarrier differentiation, the average albumin secretion rate was 2.26 µg/ml/10^6 cells, while urea was produced at 32.9 µg/ml/10^6 cells. In stirred microcarrier differentiation, albumin secretion rate was 1.77 µg/ml/10^6 cells, and urea genesis was 31.8 µg/ml/10^6 cells. CYP3A4 activity was 532 RLU/ml/10^6 cells in static differentiation and 779 RLU/ml/10^6 cells. These functional parameters were similar and not statistically different from those found in conventional 2D differentiation in tissue culture plates (Table 2). Thus, hESC can be differentiation into hepatocyte-like cells on microcarriers cultured in stirred bioreactors, reflected by the up-regulation of hepatocyte gene transcripts and functional characteristics including albumin and urea production, and CYP3A4 activity.

4. Discussion

Pluripotent stem cells including ESCs and iPSCs have been used as a cell source in directed hepatic differentiation in the past years (Hannan et al., 2013; Roelandt et al., 2012; Roelandt et al., 2010; Sancho-Bru et al., 2009; Si-Tayeb et al., 2010; Song et al., 2009). This progress raises the expectation that such stem cell-derived hepatocytes could be applied for cell transplantation or in bioartificial liver devices. Hepatocyte-like cells induced from iPSCs, which are derived from patients with different genetic backgrounds, could also be used for toxicity and metabolic studies. For these advances in stem cell differentiation protocols to be suitable for clinical or pharmaceutical use, development of robust bioprocesses that are capable of producing large numbers of hepatic cells is required. The feasibility of cultivating PSCs and their progenies in

various types of bioreactors including perfusion reactors, rotating vessels, spinner reactors and others, has already been demonstrated (Bauwens et al., 2008; Cameron et al., 2006; Fok and Zandstra, 2005; Schroeder et al., 2005). An ideal bioreactor system should be able to provide a more homogeneous culture environment and to provide for a high concentration of cells and easy access for quality control. A stirred suspension reactor system has more advantage in terms of high concentration cell culture and easy access. In suspension culture system, ESC proliferation and differentiation has been achieved as embryoid bodies (EBs). Anchorage dependent cells, such as hepatocytes, need a scaffold to attach, such as microcarriers, which can then be suspended in the culture system. Such a configuration allows the growth of anchorage-dependant cells in high density in a large-scale bioprocess.

Different types of microcarriers with various materials such as dextran and gelatin (Cytodex 1, Cytodex 3, Cultispher, Spherecol) are available for hepatic differentiation. Collagen derived beads may provide an advantage when the cells are harvested from the beads since the beads themselves can be degraded by enzymatic treatment thus avoiding a cell-bead separation step. However, gelatin beads with macro-size pores are not transparent, which makes it difficult to observe and judge the state of cells on the microcarriers during cell culture. With our differentiation protocol, hESCs attached and differentiated into hepatic cells on the different types of microcarriers. Microcarriers were coated with Matrigel to mask the collagen layer and thus promote initial hESC adhesion to the beads. Recombinant vitronectin protein coating is also commercially available and can carry hepatic differentiation of hESC when applied to microcarriers (Supplementary data Figure. 4.). This, even if vitronectin allows relatively low initial cell attachment compared to Matrigel, as it is a more defined ECM material, future studies should still investigate whether it is suitable for hESC microcarrier culture and hepatic

differentiation. Among the 4 different microcarriers, dextran beads with collagen layer supported differentiation better than other types of microcarriers, and cells could easily be judged in culture due to the transparency of the beads. As all the beads were initially coated with Matrigel masking the biological effect of the underlying microcarrier material, we cannot address whether or how the collagen layer helped the hepatic differentiation from PSCs.

The dextran microcarriers used in this study were microporous, with a void fraction exceeding 90%. As the multi-step differentiation protocol introduces the cocktails of different growth factors to the differentiating cells to drive the differentiation to designed next step, it is likely that a significant amount of medium and growth factors used during the preceding step remains inside the cell-laden microcarriers during the following step. Growth factor gradients can be formed at the near surface of microcarriers. Although we did not see a significant difference between differentiation on tissue culture plates and on microcarriers, it remains to be determined whether cytokines remaining from the previous step of differentiation may have antagonistic or positive effects on the level of differentiation, definitely when bead concentration is increased further to near industrial level. In such a differentiation bioprocess, with a higher microcarrier concentration, the effect may not be negligible anymore.

hESCs are typically passaged as colonies as hESCs do not survive well and maintain potency as single cells. However, for microcarrier culture systems, colony inoculation impedes the precise control of cell number and the even distribution of cells on the beads, which is an obstacle for developing into a bioprocess. Initial studies were done using tissue culture plates, wherein we compared seeding and expanding cells using single cell suspensions without ROCK inhibitor for a short period with seeding colonies. The differentiation outcome was comparable in both

systems (Fig. 1). This seeding system was then adopted to microcarrier culture and showed successful attachment and even distribution of cells on the microcarriers (Fig. 3A).

The microcarrier concentration used in this study was relatively low, and the culture was initiated with a relatively low inoculum of cells. The initial concentration was limited to 1 x 10^6 cells/ml, a concentration that mimics conventional 2D differentiation in tissue culture plates. We inoculated cells onto microcarriers with a temporary reduced volume to increase the microcarrier and cell concentration, thereby increasing the probability of successful contacts between cells and microcarriers during the initial 2 hours of cell attachment period. High cell and microcarrier concentration and intermittent stirring aided initial cell distribution on microcarriers and consequently affected growth kinetics during expansion of hESCs as it was determined from the cell concentration after 3 days of culture (Supplementary data Table. 1.). hESC seeded on microcarriers proliferated with similar doubling times as in tissue culture plates. hESC did not form multiple layers on microcarriers, but cell-laden beads agglomerated both in static and spinner suspension culture systems. As cells do not form multilayers on microcarriers, cell density was limited to the surface area of the total number of microcarriers. Cells expanded by 3-4 times during 2 day culture (Fig. 3). When the density reached the maximum, viable cells saturated on microcarriers, and the cell density did not increase further but was maintained. The high cell density during the initial 2 days of expansion did not affect the pluripotency markers expression of hESC.

Following 2 days of expansion, cells were induced to hepatocyte-like cells using a standard differentiation protocol in the lab (Roelandt et al., 2012). With the initial cell concentration of 1.0 x 10^6 cells/ml, differentiation in suspension culture system yielded the cell concentration of 5 x 10^5 cells/ml at the end of differentiation, which was similar to cell concentrations found in

tissue culture plates (Fig. 4). We also tested in an initial experiment whether it would be feasible to increase the initial cell and bead concentration and still achieve efficient differentiation, by increasing the initial cell concentration to 4.0 x 10^6 cells/ml. This yielded the final concentration of progeny at 2.52 x 10^6 cells/ml at the end of differentiation with comparable hepatic gene expression and albumin secretion rate (Supplementary data Fig. 1). This thus suggests the potential of larger scale differentiation in more developed and sophisticated bioprocess.

The extent of differentiation into hepatocyte-like cells on microcarriers was similar to that seen in 2D differentiation in culture dishes, as shown by qRT-PCR as well as functional assays (Fig. 4 and 5, Table 2), even if functional characteristics still remained inferior to primary hepatocytes but similar to published studies on pluripotent stem cells (Hannan et al., 2013; Yusa et al., 2011). Albumin and urea rates from the differentiated ESCs are lower than that from primary hepatocytes (Roelandt et al., 2010). CYP3A4 specific activity is also about 5 times lower than that in hepatoma cell lines (Data not shown). Only a few cells were positive for CYP3A4 and PEPCK in immunofluorescent staining (Fig. 5), in line with the qRT-PCR data, pointing to the fact that majority of cells differentiated in 2D, regardless of in culture dishes or on microbeads, do not reach a fully mature hepatocyte-like phenotype.

We also demonstrated that microcarrier differentiation is possible in a spinner culture system, yielding hepatic cells with the similar levels of hepatocyte gene expression and function (Fig. 6). The final cell concentration in the spinner differentiation was similar to that in the static culture. Cell-laden microcarriers still agglomerated together in the presence of agitation at 20-25 rpm. As shear stress cannot be avoided in stirred bioreactor cultures, the effect of shear stress on the bead surface should be examined on stem cell differentiation. Shear stress can play as a critical parameter in designing successful hepatic differentiation in a stirred bioreactor. Stirring rates

above 30 rpm did not produce a stabilized environment for hESCs on microcarriers. hESC did not remain attached to the microcarriers during the initial phase of the expansion culture. hESC induced to differentiate to hepatocyte-like cells in tissue culture plates, down-regulated their integrin (cell surface receptor that mediates cell-ECM or cell-cell interaction) expression during the first half of the differentiation culture (Supplementary Fig. 3), which is a finding that will need to be assessed further especially as it relates to the presence of shear stress produced by stirring. At physiological levels, blood flow induces shear force in the hepatic sinusoid at 20-40 dyn cm^{-2} which increases the functionality of hepatocytes (Kan et al., 2004). Recent studies have also shown that shear stress exerted on hESC-laden microcarriers induces differentiation of hPSC (Hw et al., 2011), and affects transcriptional regulation and promotion of endothelial-like cells during differentiation (Ahsan and Nerem, 2010; Mammoto et al., 2012). One method to decrease shear stress is combining microencapsulation with alginate and microcarrier culture (Serra et al., 2011).

As we did not use encapsulation, it was necessary to decrease the stirring rate below 30 rpm. This reduced stirring rate caused some agglomeration of beads forming multilayers between the beads to form semi-3D structures. Theoretically, this semi-3D structure should enhance differentiation (Nahmias et al., 2006). However, agglomeration creates a non-homogeneous differentiation environment due to various sizes of the clusters and diffusion limitation in the larger clusters. In 3D aggregation differentiation, aggregate size affects differentiated cell types (Nieden et al., 2010; Ungrin et al., 2012; Van Hoof et al., 2011), and controlling cluster size increased the efficiency of differentiating hESCs into endoderm cells (Bauwens et al., 2008; Peerani et al., 2009; Van Hoof et al., 2011).

Our study combines the proliferative characteristic of undifferentiated hESC and the high surface-to-volume ratio of microcarriers to induce larger-scale hepatic differentiation culture. This still translates the same problem that tissue culture plate differentiation of hESC faces to microcarrier culture; small population of functional hepatocytes is differentiated at the end of differentiation process. Shifting expansion phase from undifferentiated hESC to the immature hepatic cells may also amplify the efficacy of using microcarriers. *In vivo*, hepatic oval cells form the proliferative phase as transit compartment precursor cells and differentiate into several types of cells including hepatocytes and biliary cells (Fausto and Campbell, 2003). In embryonic development, bipotent hepatoblasts are similar to *in vivo* hepatic oval cells by giving rise to both hepatocytes and biliary epithelial cells. These cells express both hepatocyte (AFP, albumin) and cholangiocyte (CK7, CK19, OV-6) markers (Behbahan et al., 2011). During hESC-hepatocyte differentiation, there is a proliferative phase from day 8 to day 12 of differentiation, and some cells express *AFP* and *CK19* (Data not shown). Selecting proliferative hepatic precursor cells during the hepatic differentiation of hESC and expanding these cells on microcarriers may be able to increase the efficiency of harvesting mature hepatocytes at the end in a larger scale. However, more studies should be preceded if hepatoblasts themselves can alone be differentiated into fully functional hepatocytes without the help of surrounding other cell lineages. In addition, technically, hepatoblasts should tolerate dissociation, selection process and then seeding on to microcarriers. As much cell loss is expected after using enzymatic treatment and fastidious seeding procedure, surface property of both the cell and the bead should be carefully considered.

We conclude that microcarrier suspension culture is feasible for hESC differentiation to the hepatic cell lineage. With more careful optimization, it will facilitate the transformation of the

laboratory practice of hESC culture to scalable bioprocess generating hepatocyte-like cells at industrial scales for future clinical applications.

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